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Review Article

AN EXHAUSTIVE REVIEW ON EMERGING DRUG TARGETS OF TUBERCULOSIS WITH SPECIAL EMPHASIS ON CELL WALL SYNTHESIS

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ABSTRACT

Tuberculosis (TB) is one of the top 10 causes of mortality and morbidity. Worldwide, yet, it has been over 60 years since a novel drug was introduced in market to treat the disease exclusively. Increased number of drug resistant TB cases has prompted the search for novel potent anti-TB drug. Mycobacterial cell wall has unique structure which provides integrity to the cell. The future development of new potent anti-TB drug targets is associated with the synthesis of various cell wall constituents; the structural and genetic information about mycobacterial cell wall envelope is now available. In the present review, we have focused on prospective drug targets that can be optimum triumph for successful drug candidate.

Keywords: Cell wall synthesis, Enzymatic pathways, Mycolic acid, Tuberculosis.

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INTRODUCTION

Tuberculosis (TB), the infection caused by *Mycobacterium* species, is the major disease across the globe. There are three species of mycobacteria which are prominently observed in major population of patients. The species are *Mycobacterium tuberculosis* (Mtb), *Mycobacterium avium,* and *Mycobacterium smegmatis* out of which Mtb infection is more common. TB is one of the top 10 causes of death worldwide. According to recent World Health Organization's report, over 10 million people get infected with TB. Despite widespread use of BCG vaccine, TB infection has not been reduced as compared to other diseases [1].

The disease has reemerged as growing global health problem not only because of lack of proper therapeutic agents but also due to the development of drug resistance by mycobacterial strains. The emergence of multidrug-resistant (MDR) and extremely drug resistance (XDR) strains of this lethal pathogen renders current treatment strategies very difficult, and in some cases, there is complete failure [2]. Thus, the development of new anti-TB drug is an urgent need. The unrevealing of mycobacterial cell wall envelope has given new key to successful development of anti-TB drugs. The review describes about overview of current treatment its drawback and future prospective of drug development against TB infection.

CATEGORIES OF TB INFECTIONS

There are four categories of TB infection. Primary infection Category I belongs to new cases of sputum smear-positive pulmonary or extrapulmonary TB infection. Category II belongs to patients which are defaulted, irregularly treated, or sometimes relapsed. Category III is MDR TB where the patient is resistant to rifampicin which is given orally and needs further injectables and fluoroquinolones with combination. Category IV is extensively XDR TB which is resistant to two first-line agents (isoniazid and rifampicin), to any one fluoro-quinolone, and to any one second-line drug like amikacin [3].

TB ASSOCIATED WITH OTHER INFECTIONS

Human immunodeficiency virus (HIV)

Risk of the development of TB in HIV-infected patients is around 25 times greater than normal people due to patient's compromised immunity. The HIV-infected patients develop early resistance to the first-line TB agents due to drug interactions between medicines of both diseases. About 60% of TB cases associated with HIV-infected people results in early critical conditions with death of patient.

M. avium complex (MAC)

MAC is an atypical *mycobacterial* infection, that is, one with non-tuberculous mycobacteria which consists of two mycobacterial species *M. avium* and *Mycobacterium intracellulare*. This infection causes respiratory illness in patients, especially in immunocompromised people. It is also called as opportunistic infection, because it develops when cell-mediated immunity is markedly depressed (CD4 count drops to <50 cells/μL).

CURRENT SCENARIO OF TB INFECTION

At present, 10.0 million people around the world acquire TB disease, out of which 1 million cases occurred in infants and children. 1.6 million TB-related deaths worldwide including 0.3 million infected with HIV. Multidrug-resistant TB (MDR-TB) remains a public health crisis, the WHO estimated that there were 0.6 million new cases with resistance to rifampicin. About 82% had MDR-TB. Globally, TB incidence is declining about 2% per year, which should be accelerated to 4–5% to reduce TB risk. The summarized view of statistical data is depicted in Fig. 1.

CURRENT TB THERAPY

The current TB treatment is based on guidelines of the World Health Organization. According to clinical utility, the anti-TB drugs are divided into five groups as described below.

Group I (First-line agents)
These drugs have high efficacy as well as low toxicity they are used routinely. The drugs are isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E), and streptomycin (S).

Group II (Injectable drugs)
These are various types of antibiotics which are prescribed for MDR and XDR TB. They are administered through intravenous or other parenteral routes. Commonly used drugs are kanamycin (Km), amikacin (Am), and capreomycin (Cm).

Group III (Fluoroquinolones)
The most popular antibacterial of quinolone class are used in case of MDR-TB when patients develop resistance for first-line and second-line agents. The commonly used fluoroquinolones are ofloxacin (Ofx), levofloxacin (Lfx), moxifloxacin (Mfx), and ciprofloxacin (Cfx).

Group IV (Second-line agents)
These drugs have low efficacy or high toxicity or both and are used as reserve drugs. The drugs are ethionamide (Eto), prothionamide (Pto), cycloserine (Cs), trazodone (Trd), para-amino salicylic acid (PAS), rifabutin, and thiacetazone (Thz).

Group V (Drugs with unclear efficacy)
These drugs are used when the patient develops XDR-TB phase of infection. These are broad spectrum antibiotics such as broad-spectrum penicillins, sulfonamides, and macrolide antibiotics. Commonly used are amoxicillin/clavulanic acid, thiacetazone, clarithromycin, clofazemine, linezolid, and imipenem [4-8]. The common anti-TB drugs with their mode of action, binding site, and category are summarized as depicted below (Fig. 2 and Table 1).

The anti-TB agents are prescribed in combination as per the guidelines given by the WHO. The Directly Observed Therapy Short-course (DOTS) program is followed for the treatment of TB. The patients are grouped according to site and severity of disease, sputum smear condition, and patient history. The DOTS therapy regimen is summarized in Table 2.

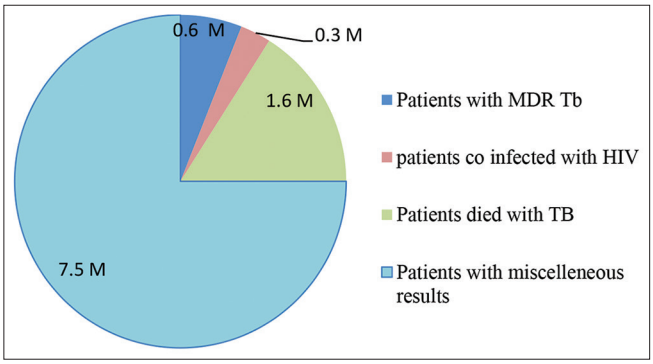

Fig. 1: Statistical evaluation of tuberculosis infection

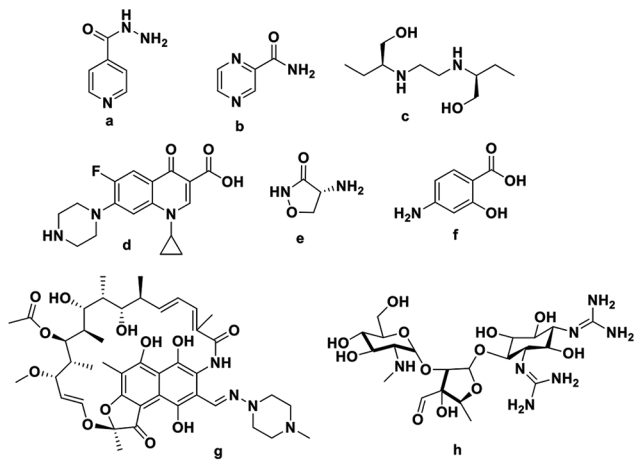

Fig. 2: Popular anti-TB drugs. (a: Isoniazid, b: Pyrazinamide, c: Ethambutol, d: Ciprofloxacin, e: D-cycloserine, f: *p*-Amino Salicylic acid, g: Ciprofloxacin, h: Streptomycin)

TARGETS OF ANTI-TB TREATMENT

Although the disease having wide range of drugs serving the treatment, still there is a need to develop new drug candidate as the current treatment is having two major problems one is discontinuation of therapy by patient due to longer duration of time and second is the development of resistance to maximum drug candidates by mycobacterial strains over the period. For the development of drugs, the research has new dimension of genomics which provide information about the nature of enzymes and metabolites of the pathogens, which will help to design scaffold for potential drug targets. To develop new anti-TB drug, mycobacterial cell envelope is attractive core. Mycobacteria have unique cell wall structure, which regulates major cell functions. The mycobacterial cell has been explored for enzymatic and genomic pathways which can be targeted for drug discovery in the past few years numerous gene targets have been explored for the anti-tubercular drug development across the globe. Out of which cell wall synthesis/cell envelope was the prime target for researchers. The targets are classified based on its presence in the cell wall [9,10]. To understand the targets, study of components of cell membrane of the mycobacteria is to be done. The mycobacterial cell wall primarily comprises arabinogalactan, peptidoglycan, and mycolic acid. Other than these components, it contains carrier proteins, carbohydrates, and lipids. The detailed structure of mycobacterial cell membrane is described in the diagram below (Fig. 3).

COMPONENTS OF MYCOBACTERIAL CELL WALL

Chiaradia *et al.* examined the mycobacterial cell membrane by transmission electron microscopy and biochemical analysis. The results determined composition of the mycobacterial cell wall. It contains more than 2100 proteins between plasma membrane and mycobacterial cell wall. Among these, the mannosyl transferase (PimB), galactofuranosyl transferase (GlfT2), Cytochrome p450, and ABC transporter (YjfF) were

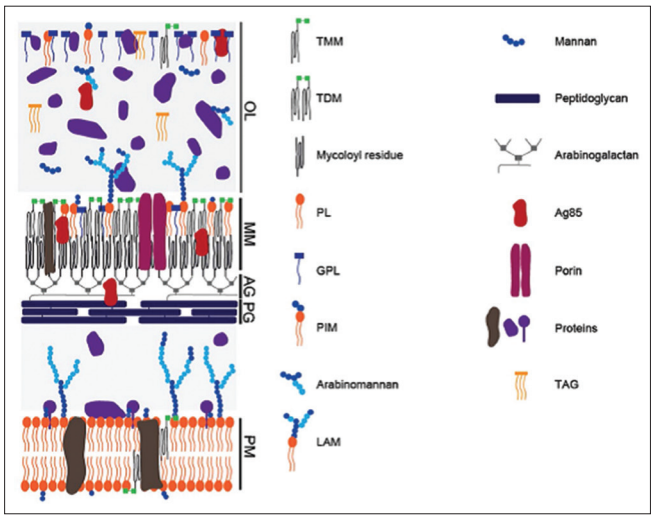

Fig. 3: Mycobacterial cell wall structure. MM: Trehalose monomycolates; PIM: Phosphatidyl-myo-inositol mannosides; GPL: Glycopeptidolipids; TDM: Trehalose dimycolates; LAM: Lipoarabinomannans; Ag85: Antigen 85; PL: Phospholipids; TAG: Triacylglycerols

Table 1: Popular anti-TB drugs with mode of action and mechanism of resistance

S. No.	Drug	Route of administration	Action against	Primary binding site and mechanism of action	Specific gene and mechanism of action	Reason to develop mechanism
First-line anti-TB agents						
1.	Isoniazid (E)	Oral, intramuscular	Extracellular and intracellular, fast multiplying bacteria	*KatG*, inhibition of cell wall synthesis (mycolic acid)	Inhibition of *InhA* and *KasA* by binding with NAD, as well inhibits mycobacterial DHFRase by binding with NADP	Mutation in *KatG*, *InhA*, and *KasA*
2.	Rifampicin (R)	Oral	Extracellular and intracellular, slowly OR intermediately dividing once	*RpoB*, interfere with mycobacterial DNA function	Interruption of RNA synthesis by binding to β- submit of mycobacterial DNA-dependent RNA polymerase	Mutation in *RpoB*
3.	Pyrazinamide (Z)	Oral	Intracellular and at inflamed sites	*pncA*, inhibition of cell wall synthesis (mycolic acid)	Get converted to pyrazinoic acid and accumulates in acidic medium, results in inhibition of mycolic acid synthesis	Mutation in *pncA*
4.	Ethambutol (E)	Oral	Intracellular and fast multiplying bacteria	*Emb AB*, inhibition of cell wall synthesis (mycolic acid)	Inhibition of arabinosyl transferases, involves in arabinogalactan synthesis thereby inhibit mycolic acid synthesis	Mutation in *embB*
5.	Streptomycin (S)	Intramuscular	Extracellular	30S ribosome, interfere with protein synthesis	Inhibition of protein synthesis prevent polysome formation, promotes disaggregation to monosomes	Decreasing affinity of binding site of ribosomes
Second-line anti-TB agents						
6.	Fluoroquinolones (FQs)	Oral, intravenous	Intracellular and extracellular	DNA gyrase A subunit, Inhibition of bacterial DNA gyrase	Binds with A subunit and interfere with strand breakdown and realizing function	Chromosomal mutation-producing DNA gyrase enzyme
7.	Ethionamide (Eto), Prothionamide (Pto)	Oral	Intracellular and Extracellular	*ethA*, inhibition of cell wall synthesis (mycolic acid)	Binds NAD+ to form adduct which inhibits InhA in the same way as isoniazid. The mechanism of action is disruption of mycolic acid	Mutation in gene producing mono-oxygenase enzyme
8.	Cycloserine (Cs)	Oral	Intracellular and extracellular	Alanine racemase, inhibition of cell wall biosynthesis (Peptidoglycan)	Inhibition of bacterial cell wall synthesis by inactivating the enzymes which racemize L-alanine and 2 D- alanine residues	Mutation in alanine racemase
9.	Para-amino salicylic acid (PAS)	Oral	Extracellular and intracellular, fast multiplying bacteria	Inhibition of folic acid synthesis	Incorporated into the folate pathway DHPS and DHFS	
Injectable agents						
10.	Kanamycin (Km), amikacin (Am)	Intravenous	Intracellular, macrophages, and extracellular	30S-50s ribosome, interfere with protein synthesis	Inhibition of protein synthesis prevent polysome formation, promotes disaggregation to monosomes	Decreasing affinity of binding site of ribosomes

most abundant in the plasma membrane. Plasma membrane also contains lipoglycans, phospholipids including phosphatidylinositol mannosides, and other glycolipids [11]. Antigen 85 complex proteins, porins, putative transporters, and mammalian cell entry protein family were mostly found in *mycobacterial* cell wall fraction that contains mycolic acid esterifying arabinogalactan constituting the inner leaflet of mycomembrane, along with glycolipids, phospholipids, lipoglycans, and proteins [12].

The cell wall of *mycobacteria* contains outermost layer (OL) which constitutes proteins with carbohydrate and negligible amount of lipids. After the OL layer of mycolic acid, mycomembrane (MM), arabinogalactan (AG), and peptidoglycan (PG) are present, respectively. The inner leaflet of the MM is made of very long-chain fatty acids (mycolic acids) esterifying (AG), covalently attached to (PG). The outer leaflet of the MM is presumably composed of lipids extractable with organic solvents, which includes phospholipids, trehalose mycolates, glycopeptidolipids, and lipoglycans. A periplasmic space separates the cell wall from the conventional lipid bilayer of plasma membrane (PM) of phospholipids and proteins. The cell envelop has different components regulated by various enzymes. The enzymes are primary targets for cell wall inhibition. Mycolic acid metabolism is considered as upcoming target related to mycolic acid [13].

Table 2: Category wise treatment regimens for TB

Category	Intensive phase	Continuous phase	Duration (months)	Comment
I New patient	2$ HRZE daily	4$ HR daily	6$	Optimal
	2 HRZE daily	4$ HR thrice weekly	6	Acceptable if DOT ensured
	2 HRZE thrice weekly	4$ HR thrice weekly	6	Acceptable if DOT ensured
II Previously treated patients pending DST result	2 HRZES daily + 1 HRZE daily	5 HRE daily	8	For patient with low/medium risk of MDR-TB (failure, default)
	Empirical£ (standardized) MDR-regimen	Empirical (standardized) MDR-regimen	18-24 Or till DST results	For patients with high risk of MDR-TB (failure, 2nd default, contact of MDR-TB)
III MDR-TB	6-9 Any 4 of Km, Ofx/Lfx, Eto, Cs, Z, E, PAS + Pyridoxine 100 mg/day	18 Any 4 of Ofx/ Lfx, Eto, Cs, E, PAS + Pyridoxine 100 mg/day	24-27	For patients with failure to 1st line and 2nd line treatment
IV XDR-TB	Group V drugs are prescribed according to the severity of infection for unpredicted period of time.			

DST: Drug sensitivity testing; DOT: Directly observed therapy; $- The numerals indicate duration of phase, £- Empirical (standardization) MDR regimen is country depending on local data and situation.

Peptidoglycan biosynthesis

Peptidoglycan is the essential cell wall component of *mycobacterial* cell wall which gives rigidity to cell. Peptidoglycan consists of N-Acetyl Glucosamine (NAG), N-Glycolyl muramic acid (NAM), and alanine (Al). The peptidoglycan biosynthesis is targeted for anti-TB drug development from decades but still any drug candidate other than D-clycoserine has not shown promising effect, D-cycloserine inhibits peptidoglycan biosynthesis by inhibiting essential enzymes alanine racemase (Alr) and D-Ala-D-Ala ligase. It was predicted that bacitracin antibiotic also targets peptidoglycan biosynthesis but exact mechanism is not known. Another target in peptidoglycan synthesis is glutamate racemase for which β-chloro-D-alanine and its derivatives are under study, the exact mechanism is not identified but the enzyme is considered as potential target. The peptidoglycan biosynthesis can be inhibited by alanine like small molecules which can act as competitive inhibitor of alanine inhibiting attachment of alanine to NAG and NAM to form peptidoglycan [14-18].

Arabinogalactan biosynthesis

The arabinogalactan layer is present just next to the peptidoglycan layer. It contains sugars like rhamnose, galactose, phospholipids, and carrier proteins. The next important target in the mycobacterial cell wall is arabinogalactan synthesis. Ethambutol is the one of the extensively used anti-TB drugs which act through inhibition of arabinogalactan synthesis through inhibition of arabinosyltransferase enzyme.

Uridine diphosphate-galactopyranose (UDP-galactopyranose) is also identified as important enzyme in this pathway which involves in attachment of galactose in the arabinogalactan. Recent studies reported that the biosynthesis of the cell wall galactan of mycobacteria through Rv3808c protein can be inhibited through galactosyl transferase enzymes. Decaprenyl phosphorylase-2-epimerases are challenging target in the mycobacterial synthesis because targeting this enzyme has many challenges due to its oxidoreductive property responsible for degeneration of chemical structure of drug. A prodrug approach toward the drug development can be considered as novel way to target the enzyme, benzothiazinone which inhibits the enzyme and is under clinical investigation. From the description of enzymes, one can predict small cyclic molecules with structural similarity to galactose with hetero-atoms as potent candidates to target the pathway [19-21].

Mycolic acid biosynthesis and metabolism

Mycolic acid is unique feature of mycobacteria it is fatty acid containing carbon chain of 70 carbons. It makes the mycobacteria more resistant to chemical damage and dehydration. It also helps mycobacteria to grow inside the macrophages by effectively hiding from host immune system. There are four types of enzymes related to mycolic acid:

1. Fatty acid synthesis I (FAS I): Ketoacyl-ACP synthases, Catalase-peroxidase
2. Fatty acid synthesis II (FAS II): ENR enoyl acyl carrier protein reductase, EthA gene
3. Fatty acid synthesis condensing enzymes: polyketide synthase (Pks13) and fatty acid desaturase (FAD13/FAD32)
4. Mycolic acid metabolism: Mycobacterial membrane protein large (mmpL), mycobacterial membrane protein small (mmpS).

Fatty acid synthesis I pathway (FAS I)

The pathway includes synthesis of C_{26} straight chain fatty acids, which involves enzymes such as Ketoacyl-ACP synthases (CasA) and Catalase-peroxidase (KatG). The enzymes are inhibited by popular drugs such as isoniazid, pyrazinamide, and ethionamide. The drugs are susceptible to resistance due to mutations in genes regulating the enzyme. The ketoacyl-ACP synthases enzyme is indirectly involved in mechanism of many drugs but direct target for the enzyme is under study. The KatG is involved in activation of isoniazid into isoform to the anion of nicotinic acid, which further inhibits the cell all synthesis by inhibiting FAS II pathway competitively. FAS I pathway can be inhibited by chemical moieties such as thiazolidine linezolid and heterocyclic five and six-membered derivatives [22,23].

Fatty acid synthesis II pathway (FAS II)

The pathway further involves in chain elongation and addition of functional groups to the chain. It involves enzymes such as enoyl acyl carrier protein reductase (InhA) and EthA gene. The well-known drug isoniazid inhibits (InhA) enzymes after activation by KatG. EthA gene is another target on the FAS II pathway which is inhibited by ethionamide. To inhibit FAS II pathway, there are many chemically and biologically important scaffolds such as epigallocatechin gallate, luteolin, cinnamic acid derivatives, quinones, thiocarbamides, and diphenyl ether [24,25].

Fatty acid synthesis condensing pathway

The pathways involve condensation and attachment of mycolic acid to arabinogalactan layer. The enzymes involved in pathway are polyketide synthase (Pks13) and fatty acid desaturase (Fad13/Fad32). The pathways are not having any current potential anti-TB targets. However, there are numerous drugs and enzyme pathways such as acyl-adenylate monophosphate ligase (acyl-AMP ligase), FadD32, acyl-coenzyme A (CoA) carboxylase. Benzofuran, thiophene, and coumarin derivative inhibit the fatty acid synthesis condensing pathway [26].

Mycolic acid metabolism pathway

The mycolic acid metabolism is indirectly involved in mechanism of many antitubercular drugs and has important function of the drug influx

Table 3: Summary of enzyme targets in mycobacterial cell wall synthesis

S. No.	Target (Gene/ Enzyme)	Specific target	Mechanism of target enzyme	Drug target synthesized/ in use	Mechanism of drug	Novel target identified drug/ enzyme	Scaffolds under research/clinical trials
1.	Peptidoglycan biosynthesis	Alanine racemase (Alr), D-Ala-D-Ala ligase	Catalysis of first 2 steps of Peptidoglycan biosynthesis	D-cycloserine, bacitracin,	Inhibition of bacterial cell wall synthesis by inactivating the enzymes which racemize L-alanine and 2 D- alanine residues	Alanine like small molecules	--
		Glutamate racemase	----------UNDER STUDY----------				β-Chloro-D-Alanine
2. 1.	ARABINOGALACTAN BIOSYNTHESIS	Arabinosyl transferases (EmbA, EmbB, EmbC)	Formation of the terminal hexaarabinofuranoside portion of arabinogalactan, where mycolic acid get attached	Ethambutol	Inhibition of arabinosyl transferases, involves in arabinogalactan synthesis, thereby inhibit mycolic acid synthesis	UDP-galactopyranose	
		Galactosyl transferases (Galf)	Biosynthesis of the cell wall galactan of mycobacteria through Rv3808c protein	----------UNDER STUDY----------			
		Decaprenyl phosphorylase-2-epimerase (DprE1/DprE2)	Arabinofuranose donor in biogenesis of AG and LAM	---	Inhibition of (DPA) formation	Dinitrobenzamide derivatives	Benzothiazinone
3.	Mycolic Acid Biosynthesis FAS-I pathway	Ketoacyl-ACP synthases (KasA, KasB)	Synthesis of C16-C26 chain of Fatty Acid (Mycolic Acid) Biosynthesis	----------UNDER STUDY----------			
		Catalase-peroxidase (KatG)	Activation of NAD^+ enzyme by electron donation	Isoniazid	Inhibition of *InhA* and *KasA* by binding with NAD, as well inhibits mycobacterial DHFRase by binding with NADP	Linezolid, thiazolidine derivatives	---
4.	Mycolic acid biosynthesis FAS-II pathway	ENR enoyl acyl carrier protein reductase (InhA)	Catalysis of extension of fatty acid chain up to C56	Isoniazid (ENR subunit of InhA)	Inhibition of *InhA* and *KasA* by binding with NAD, as well inhibits mycobacterial DHFRase by binding with NADP	*FabH, MabA,* Epigallocatechin gallate luteolin cinnamic derivatives quinones diphenyl ether	---
		Flavin adenine dinucleotide (FAD)-containing Baeyer-Villiger monooxygenase (EthA)	Catalysis of extension of Fatty Acid Chain up to C56	Ethionamide	Inhibition of InhA	Thiocarbamide derivatives	----

(Contd...)

Table 3: ***(Continued)***

S. No.	Target (Gene/ Enzyme)	Specific target	Mechanism of target enzyme	Drug target synthesized/ in use	Mechanism of drug	Novel target identified drug/ enzyme	Scaffolds under research/clinical trials
5.	Mycolic acid biosynthesis FAS condensing enzyme	polyketide synthase (Pks13)	Catalysis of the final condensation step in mycolic acid biosynthesis	---------UNDER STUDY---------		acyl-AMP ligase, Benzofuran; thiophene Coumarins	---
		Flavin adenine dinucleotide (FadD13/ FAD32)	Part of fatty acid acyl ligase (FAAL) which adenylate Fatty acid long chain which is transferred to pks13	----------UNDER STUDY-------		Coumarin derivatives	Fatty acid derivatives
6.	Mycolic acid metabolism	MmpL and MmpS	Mediate transport of important cell wall lipids across the mycobacterial membrane.	Indirect involvement in transport of drug molecules	-----	---	SQ109

in *mycobacteria.* The acid associated pathways can be explored through different scaffolds such as cinnamic acid, thiazolidine, rhodanine scaffolds, and many other natural products [27-37]. Summary of enzyme targets is depicted in Table 3.

ANTI-TB SCAFFOLDS IN PIPELINE

The era of anti-TB drug development is rapidly changing from phenotypic to genotypic research. Till date, numerous scaffolds are explored for this purpose. The scaffolds screened are ranging from NCE's obtained from natural sources, biological extracts, and enzymes from genetically modified microorganisms and from synthetic sources. In the past 10 years, various scaffolds from synthetic source such as β-chloro-D-alanine, ethers, dinitrobenzamide (benzothiazinone), linezolid, thiocarbamides, cinnamic acid, benzofuran, and quinazolinones derivatives are used. Few natural products such as epigallocatechin, quinines, and coumarins are also explored for the development of new drug.

CONCLUSION

Today, it is very important to develop new anti-TB drugs. With the help of enzymatic and genetic pathways, we can achieve target-based drug development which will be helpful to overcome the problems associated with existing anti-TB therapy. The unique structure of *mycobacterial* cell wall makes it ideal for target-based drug development. The architecture of the mycobacterial cell wall is complex and represents a substantial permeability barrier that is made up of a wide variety of compounds including mycolic acids, free lipids, polysaccharides, glycolipids, and lipoarabinomannan. Many of these components are genera or species specific. Most of the existing anti-TB drug candidates targets cell wall to inhibit bacterial growth. Nevertheless, none of the known cell wall synthesis inhibitors are endowed with rapidly acting sterilizing activity, hence, these drugs are urgently needed in TB control programs. All apparently require actively replicating bacteria for efficacy. A problematic issue one addresses latent TB, a condition in which mycobacteria are not actively dividing or synthesizing cell wall component which is apparently prevalent in about one-third of the global population. At present, the anti-TB drug development should be focused on potent drug candidates who can withstand to MDR TB. The novel drug development includes targeting unexplored cell wall components for drug development, shortening duration of existing drug therapy, development of potent anti-TB drugs, and repurposing of old drugs.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this research article.

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